Plant Breeding **126**, 323—328 (2007)
doi:10.1111/j.1439-0523.2007.01362.x

Cytological behaviour of the somatic hybrids *Passiflora edulis* f. *flavicarpa* + *P. cincinnata*

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With 2 figures and 3 tables

Received June 29, 2006/Accepted November 16, 2006
Communicated by J. Grunewaldt

Abstract

Meiosis and pollen viability of interspecific somatic hybrids, i.e. *Passiflora edulis* f. *flavicarpa*, the yellow passion fruit + *P. cincinnata*, a wild species, were examined and compared with their corresponding diploid fusion parents. Firstly, the cybrid nature of these hybrids was confirmed by leaf-stomatal measurements and RFLP analyses using four mitochondrial genes as probes. The meiotic behaviour revealed relatively high stability, with most of the hybrid cells showing 18 bivalents. Some instability, such as a quadrivalent configuration was also recorded which has been interpreted as an interchange that occurred in the progenitors more than as a result of *in vitro* culture or chromosome reorganization in the new genome. Even in low frequencies, the occurrence of univalents resulted in mis-division, laggard and micronucleus formation. High values of pollen viability (> 70%) were found in the diploid parents as well as in the hybrid plants. The results are discussed in relation to the possibilities of applying somatic hybridization for improving passion fruit varieties.

Key words: *Passiflora* — somatic hybrids — meiotic analyses — polyploids — yellow passion fruit — RFLP — mitochondrial genes — stomatal measurements

Passiflora edulis f. *flavicarpa*, the yellow passion fruit is one of the important fruit crops in Latin America where many species of *Passiflora* are native to both tropical and sub-tropical climates. The yellow passion fruit has an ovoid-shaped fruit with a very strong exotic flavour, and a bright orange colour. It is consumed as fresh fruit, being found in the majority of Brazilian and Andean villages, and it gives a juice that is processed industrially. Although small-scale, the juice is manufactured to be exported to EU countries.

Passion vines are susceptible to the bacterium *Xanthomonas axonopodis* pv. *passiflorae*, which in some recent years has resulted in great losses to the juice industry and fruit producers, mostly in southeastern Brazil. Infections are characterized by brownish lesions on the leaves and fruit, and the disease progress usually causes defoliation of the vines in commercial orchards. Some of the accessions of the wild relatives such as *P. cincinnata* are an unexploited germplasm reservoir, which possess tolerance to the bacterial disease and to *Phytophthora* sp., the source of collar and root rot in various tropical regions (Junqueira et al. 2005). Both are severe diseases obliging producers to replace yellow passion fruit plantings every 24–35 months. In order to introduce resistance genes to aerial and soil borne plant diseases both sexual and somatic hybridizations are currently being tried, as well as genetic transformation technologies (for a review see Vieira and Carneiro 2004).

Somatic hybrids of *Passiflora* have been developed here between *P. edulis* f. *flavicarpa* and *P. amethystina*, and *P. edulis* f. *flavicarpa* and *P. cincinnata*. Identification was performed at the callus phase by isoenzyme and total protein profiles, i.e. before shoot regeneration, and the plants obtained were vigorous and male fertile (Vieira and Dornelas 1996, Barbosa and Vieira 1997). However, the main problem in the practical use of those somatic hybrids for gene introgression lies in their fertility and chromosomal instability. Thus, research in this area is very important because it provides the means for characterizing the meiotic behaviour and fertility of the hybrid plants.

The formation of natural allopolyploids requires the adaptation of two nuclear genomes within a single cytoplasm, which may involve programmed genetic changes during the first generations following genome fusion (Lukens et al. 2006). Here, morphological, molecular and cytological analyses of four somatic-hybrid plants between *P. edulis* f. *flavicarpa* and *P. cincinnata* carried out to confirm their hybrid nature, and to evaluate their cytogenetic behaviour in comparison with parental diploids are reported.

Materials and Methods

Plant Materials: The yellow passion fruit (*Passiflora edulis* Sims f. *flavicarpa* Degener) [E] and the wild species *Passiflora cincinnata* Masters [C], both 2n = 2x = 18, and the following somatic hybrids denoted as [E + C] #07, #14, #25 and #26 (2n = 4x = 36) were studied. Briefly, the hybrid cells were obtained by PEG-mediated protoplast fusion and shoot regeneration via indirect organogenesis. Selection was performed based on total protein and isoenzyme electrophoretic patterns at the callus stage (Vieira and Dornelas 1996). After being acclimatized, the plantlets were transferred to the field. They were planted in rows 3 m apart, tied, and supported on wire trellises up to 1.8 m high. Row-spacing of 2 m was adopted. The vines grew to maturity and flourished yearly from spring to autumn, i.e. from end of October to May but did not set normal fruits, even when artificially crossed.

Confirmation of polyploidy by leaf stomata analysis: Young leaves were collected from new shoots from each of the hybrid plants and the parental genotypes. A piece of the lower epidermis was placed on glass slides, covered with glass coverslips and analysed under the optical microscope (40×, Olympus BX50, Olympus Optical Co., Ltd., Tokyo,

Japan) by using a micrometer eyepiece. The number and area (length × width) of open stomata were examined in a sample of 100 and 50 microscopic fields, respectively. The Tukey-test (SAS Institute Inc SAS/STAT 1988) was used to discriminate the parental forms from the hybrids.

Confirmation of the cybrid nature by RFLP analyses: Total DNA was extracted from leaf tissues of adult plants (2 g) in CTAB-extraction buffer. The mixture was incubated (65°C for 1 h), then chloroform/isoamyl alcohol (24 : 1) was added and the tube centrifuged (1000 g, 10 min). This procedure was repeated, and RNAse (10 μg/μl) was added to a tube containing the upper phase. After incubation (2 h, room temperature), isopropanol was added to allow DNA to precipitate out. After lifting out the DNA with a glass hook, it was treated with Wash-1 solution (76% aqueous ethanol; 0.2 mM sodium acetate) for 20 min, and with Wash-2 solution (76% aqueous ethanol; ammonium acetate 10 mM) for 5 min, allowed to air-dry before dissolving in TE buffer. DNA quantification was achieved after standard gel electrophoresis.

Samples of DNA (5 μg) were digested separately with 30 U of *Bam*HI, *Bgl*II, *Eco*RI and *Hin*dIII in 30 μl buffer (10x) plus 7.5 μl spermidine (0.1 M). The mixture was diluted in water to a final volume of 300 μl. Reactions were performed overnight at 37°C. Then, 30 μl of 3 M sodium acetate (pH 5.2) plus 900 μl absolute ethanol were added, and DNA precipitation occurred after 2 h (−70°C). After centrifugation (15 min, 9000 g at 4°C) and washing (500 μl of cooled 70% aqueous ethanol), the pellet was allowed to air-dry (4 h) before dissolving in TE. The product was submitted to electrophoresis (35 V) in 0.8% agarose gels prepared with TAE buffer (1x) for 18 h. Then, the fragments were depurinized for 15 min in 0.25 M HCl, the gel washed with distilled water and placed in a denaturation solution (0.5 M NaOH, 1.5 M NaCl), shaken slowly for 30 min. The gel was rinsed and treated with 1.5 NaCl/1 M Tris-HCl (pH 7.5).

Subsequently, the fragments were blotted on to a nylon membrane (Hybond N+; Amersham, Amersham International, Buckinghamshire, UK) for 18 h and then hybridized. The probe (25 ng) was labelled with alpha-32P [dCTP]-10 mCi/ml by a random primer method using the Prime-a-Gene labelling kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Plasmids bearing inserts of maize-mtDNA were used for obtaining heterologous gene-specific probes (*atp*α; *atp6; cox1* and *cob2*) that were used for analysing the organellar constitution of the parental and somatic hybrid plants. Bands were visualized by autoradiography (Kodak Biomax, XAR-5 film, Rochester, NY, USA).

Preparation of the slides: Flower buds were collected from the vines between 8 and 10 AM, and kept in the fixative (96% ethanol : glacial acetic acid, 3 : 1) for 24 h at room temperature. Buds were kept in 70% ethanol (v/v) at 5°C. The slides were prepared by smearing a piece of anther in 40% acetic acid and then staining the microsporocytes with 2% propionic carmine. All the meiotic phases were studied. At least 40 cells in diakinesis were analysed for each of the hybrid plants. Pollen grain viabilities were also analysed, according to Alexander's protocol that employs malachite green and fuchsinic acid (Alexander 1980).

Results

Confirmation of the hybrid and cybrid nature

The parental species showed similar stomatal numbers and areas, which were different from the somatic hybrid values. The ploidy level interferes with the number and size of stomata, but the four hybrid plants did not show the same pattern (Table 1).

Half of the 16 probe-enzyme combinations were informative and showed a good DNA profiling. Results provided evidence that the four somatic hybrids have a cybrid nature. The hybrid plants inherited alleles from one and/or other parent, depending on the mtRFLP locus examined. For example, the profile of the wild species, i.e. *P. cincinnata*, which was used as the callus-derived parent during fusion procedures (Vieira and Dornelas 1996), was identified in all hybrids (Fig. 1a,b). The allele from the cultivated species is shown in the pictures (c) and (d) (Fig. 1) in the somatic hybrids #7, #25 and #26. Interestingly some of the enzyme/probe hybridizations revealed additional novel bands or alleles (Fig. 1e). The hybrid #14, for instance, tends to show the *P. cincinnata* profile.

Table 1: Area and number of leaf stomata per microscope field (40×) of *P. edulis* f. *flavicarpa* [E] and *P. cincinnata* [C] (2n = 2x = 18) and their four somatic hybrids [E + C] (2n = 4x = 36)

Plant material	Area (μm^2)	n
[E]	20.4 ± 0.7 **a**	7.0 ± 0.3 **a**
[C]	21.1 ± 1.4 **a**	6.6 ± 1.5 **a**
[E + C] #14	25.4 ± 0.7 **c**	4.9 ± 0.7 **b**
[E + C] #25	24.2 ± 0.7 **c**	5.0 ± 1.3 **b**
[E + C] #07	28.4 ± 5.8 **b**	5.4 ± 0.6 **c**
[E + C] #26	32.6 ± 5.7 **b**	5.7 ± 0.6 **d**

Means followed by same letter (in bold) are statistically different according to the Tukey-test at P = 0.05.

Cytological analysis of diploid parents and somatic hybrids

As expected, the parental cells at diakinesis showed the presence of nine bivalents (Fig. 2a,b) and the course of meiosis was regular. Chromosome bridges and laggards were observed, possibly as a consequence of the formation of univalents, though they were not actually recorded. These abnormalities occurred at a very low frequency, i.e. < 2% of the cells (Table 2).

In *P. edulis* f. *flavicarpa*, two bivalents were attached to the nucleolus (Fig. 2a) in 67% of the cells at diakinesis, suggesting the presence of at least two pairs of active 45S rDNA loci. In *P. cincinnata*, this percentage was higher (73%).

The four hybrid plants were 2n = 4x = 36; the hybrid cells *per se* were not variable in number, i.e. no aneuploid cells occurred, at least within the sample cells analysed. (Fig. 2c). These results agree with chromosome counting performed in somatic metaphases reported previously (Dornelas et al. 1995).

A certain frequency of laggards and anaphase bridges in both divisions was observed in all hybrid plants (Table 2; Fig. 2d–f). However, differences amongst them were noted: [E + C] #07 and #25 had a more irregular meiotic behaviour than [E + C] #14 and #26, in which, on average, 24.4% and 19.60% of the cells at meiosis I showed laggards and anaphase I bridges at equal frequencies, respectively. In the second division, the configuration changed, and the hybrids #25 and #26 had more irregular behaviour with 16.2% and 13.3% of the cells showing laggards and anaphase bridges, respectively.

The frequency of bivalents at diakinesis in the hybrids varied from 55% to 78% (Table 2; Fig. 2c). These percentages should be associated with the presence of a quadrivalent, which was found in 30.0% and 32.5% of the cells of the hybrids #07 and #26, respectively (Fig. 2f), and in 17.1% and 13.3% of the cells of the hybrids #14 and #25, respectively (Table 2). The disassociation of those quadrivalents should explain the laggards and the chromosome bridges observed at division II (Table 2).

The hybrids #07 and #25 showed higher percentages of univalents, which were also the plants that had cells with higher proportions of laggards at meiosis I and II (Table 2). The occurrence of univalents varied from 4.2% in [E + C] #14

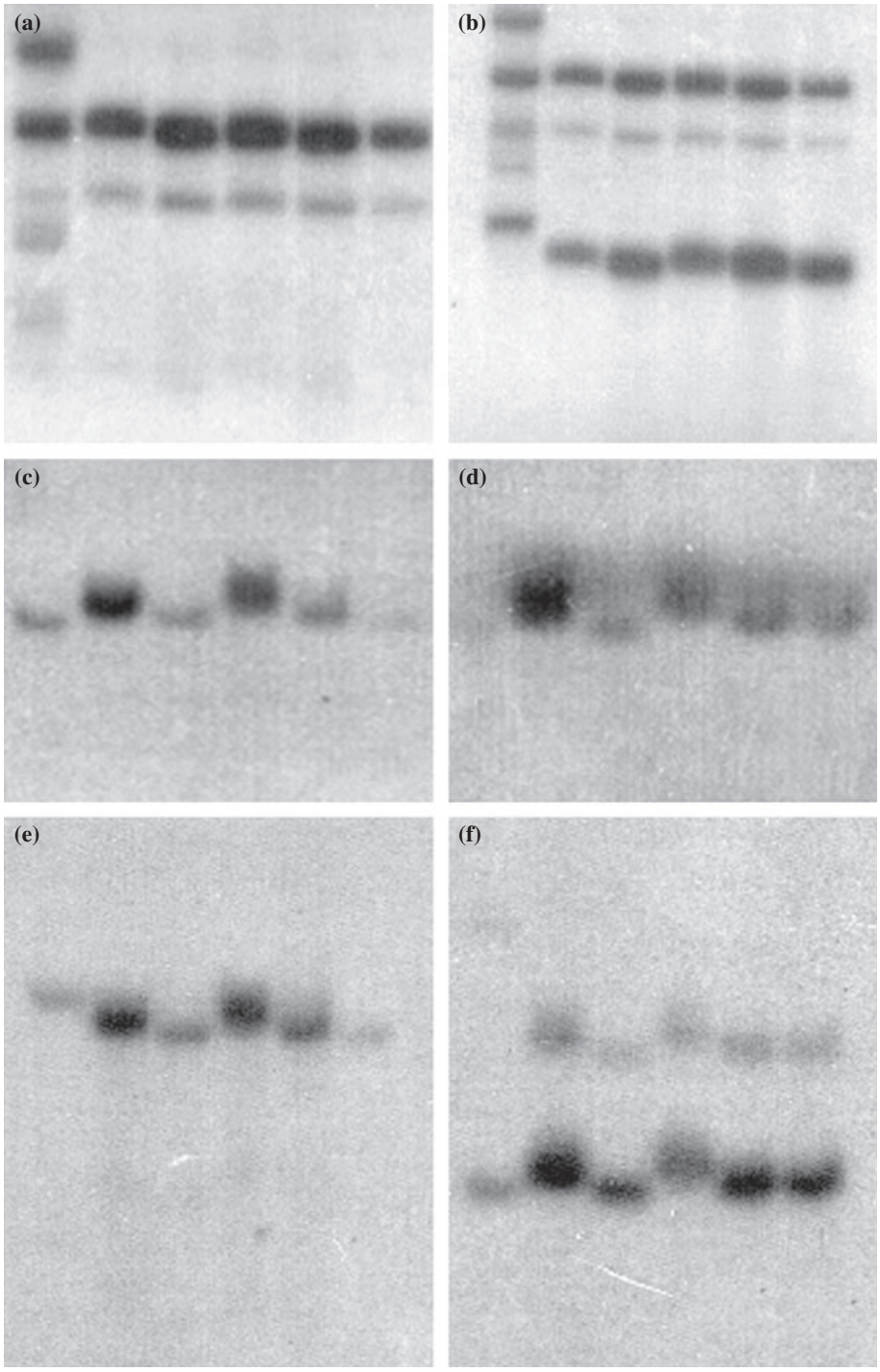

Fig. 1: Southern blot hybridization with the mitochondrial gene probes: *atp*6 (a, c, d) *atp*-α (b) and *cox1* (e, f). The DNA was digested with *Eco*RI in c and e; with *Bam*H1 in a and b, and with *Hin*dIII in d and f. The patterns of *P. edulis* f. *flavicarpa* and *P. cincinnata* are shown in the first and second lanes, respectively. The profiles of the somatic hybrids [E + C] 7, #14, #25 and #26 are shown consecutively from the third to sixth lane

up to 15% in [E + C] #07 and #25, being correlated with other meiotic alterations and as well bivalent frequency at diakinesis. In the plant [E + C] #25 with 15% of diakinesis cells showing univalents, they appeared as laggards at metaphase I (12.3%) and underwent centromere misdivision (2.3%). Moreover, the results suggest that chromosomes that suffered longitudinal division at meiosis I underwent centromere misdivision at meiosis II.

The frequency of microspores with micronuclei was low considering the abnormalities found at all the meiotic stages (Table 2). Probably, many laggards forming bridges at anaphase I and II were included in telophase nuclei. Certainly, a fraction of laggards was included as entire chromosomes.

The frequency of laggards in metaphase II cells was somewhat the same in metaphase I (Table 2). However, misdivision was five times more frequent at metaphase II, although laggards were found in a reduced frequency (three times less), suggesting that almost all the chromosomes that underwent longitudinal division at first division did undergo misdivision at meiosis II. Laggards in [E + C] #25 were observed in 7.6% of the anaphase II cells and did result in a micronucleus in 8.7% of the tetrad cells. Those chromosomes that went through longitudinal division at first meiosis underwent misdivision because they were attached to the centromeres, while laggards at anaphase II were eliminated.

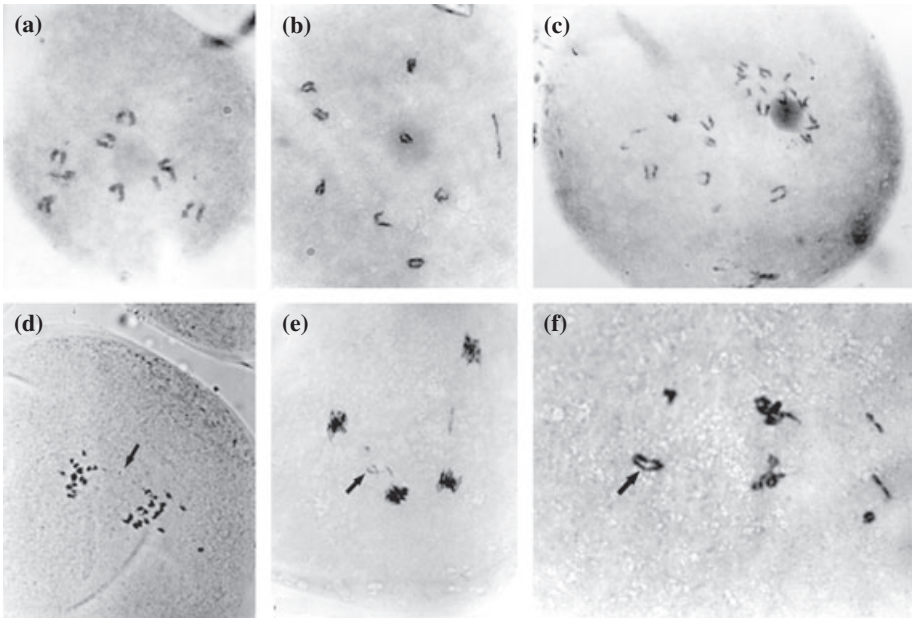

Fig. 2: Diakinesis configurations: 9 II in *P. edulis* f. *flavicarpa* (a) and *P. cincinnata* (b) and 18 II in the hybrid cell (c). Note one (b) and two (a) bivalents attached to the nucleolus. A bridge-like structure derived from a univalent that mis-divided in anaphase I (d). Laggard chromosomes (arrowed) in two anaphase I cells: note the rod laggard chromosome (e). A quadrivalent at diakinesis is shown in f (arrowed)

Table 2: Meiotic behavior (in percentages) in *P. edulis* f. *flavicarpa* [E], *P. cincinnata* [C] and their four somatic hybrids [E + C]

	Diakinesis configurations					Laggards			Laggards				
Plant material	9 II	18 II	17 II + 2 I	16 II + 1 IV	No. cells	MI	AI	Bridges	MII	AII	Chromatid bridges	Tetrads with micronuclei	No. tetrads
[E]	100.0	—	—	—	40	1.0	0.0	0.6	0.4	0.0	0.2	—	—
[C]	100.0	—	—	—	40	1.3	0.0	0.2	0.0	0.8	0.6	—	—
[E + C] #07	—	55.0	15.0	30.0	40	19.9	28.8	1.2	15.8	16.0	1.8	23.2	524
[E + C] #14	—	78.5	4.2	17.1	70	7.1	28.2	5.0	13.0	4.0	10.6	6.4	827
[E + C] #25	—	71.6	15.0	13.3	60	12.3	26.9	2.3	15.5	7.6	16.1	8.7	935
[E + C] #26	—	60.0	7.5	32.5	40	15.3	15.5	2.1	13.4	13.4	13.3	17.6	631

MI and MII, first and second metaphase, respectively.
AI and AII, first and second anaphase, respectively.

Table 3: Percentage of pollen viability (diagonal) and t-test results for the comparison of mean pollen grain viabilities of *P. edulis* f. *flavicarpa* [E], *P. cincinnata* [C] and their four somatic hybrids [E + C]

	[E]	[C]	[E + C] #07	[E + C] #14	[E + C] #25	[E + C] #26
[E]	96.12 ± 2.06	0.65	17.77**	20.57**	24.53**	13.98**
[C]		96.77 ± 0.93	18.42**	21.22**	25.18**	14.63**
[E + C] #07			78.35 ± 7.53	2.80	6.76**	3.79
[E + C] #14				75.55 ± 5.09	3.96*	6.59*
[E + C] #25					71.59 ± 2.06	10.55**
[E + C] #26						82.14 ± 7.60

Significant at P-values: *0.05 and **0.01.

Analysis of pollen viability (V) and the t-test for mean comparisons are presented in Table 3. Parental V-values were approximately 96%. In the hybrid plants, on average, all values were >70%. However, the hybrid plants [E + C] #07 and #14 had similar V-values but these were different from those of [E + C] #07 and #26.

Discussion

The procedures used here confirmed the hybrid nature of the protoplast fusion-derived plants. The four hybrids did not show the same pattern in terms of the number and area of stomata as well as in the RFLP profiles. Cell differentiation is regulated at the molecular and cellular level, but patterning of stomata is not an independent event, resulting from the interaction of spatial organization in a field of cells (see Bean et al. 2002 and references therein). This organ-level viewpoint may possibly explain the differences observed here.

In the Southern analysis, the hybrids had an identical RFLP profile to either one of the parents, depending on the locus analysed. Novel RFLP patterns were also detected. As reported in *Solanum* spp. (Xu et al. 1993) the results clearly show that mtDNA rearrangements occurred in the somatic hybrids.

For the meiotic analyses, one can first state that the two bivalents attached to the nucleolus of the parental cells

correspond to the smallest chromosomes, i.e. 8 and 9. A previous study on *Passiflora* using fluorescent *in situ* hybridization detected positive signals corresponding to 45S rDNA sites on the secondary constrictions of chromosome 8 and satellites of chromosome 9 of *P. edulis* f. *flavicarpa* and *P. cincinnata* (Cuco et al. 2005). In addition, chromomycin A3 bandings were found in regions corresponding to 45S rDNA sites, therefore, in two chromosome pairs. No other preferentially GC-rich regions were observed in these species. The nucleolar activity was also investigated by silver staining and were four silver-positive signals were detected on the smallest chromosomes, i.e. 8 and 9.

One may speculate that pairing between the smallest chromosomes, which is much conserved in the subgenus *Passiflora*, resulted in the quadrivalent formation. These four homeologous chromosomes may pair as two bivalents or as a quadrivalent. A chromosomal translocation could be the reason for the quadrivalent configuration, originating from a chromosomal rearrangement between the parental species, which is only observed when both genomes are put together, e.g. when somatic hybrids are created. It is less likely that a translocation could be a consequence of the protoplast fusion itself, *in vitro* stress, and the hybridization process.

Minimal differences are also reported between the karyotypes of *P. edulis* f. *flavicarpa* and *P. cincinnata* (Cuco et al. 2005). Chromosome lengths are very similar, as there is a perfect synteny with respect to the location of the genes 18S-5.8S-26S and 5S. In addition, the DNA content amongst the *Passiflora* species (at least into the subgenus *Passiflora*) is slightly variable (Souza et al. 2004), and *P. edulis* f. *flavicarpa* and *P. cincinnata* are very close in terms of phylogenetics (Muschner et al. 2003, Padua 2004). Based on all those features, high chromosome stability is not expected for the somatic hybrids studied here.

Due to bivalent frequency, the hybrids tend to show a diploid-like behaviour, and gene introgression should occur preferentially by chromosome substitution, along with the subsequent backcrossings of the hybrid offspring with the cultivated parent. The presence of two complete diploid sets of chromosomes should favour homologous pairing, diminishing the occurrence of homeologous chromosome synapsis. Another reason for chromosomal stability would be the expression of a *Ph1*-like allele, firstly described in wheat. The *Ph1* locus ensures that homologous and not homeologous chromosomes synapse and undergo reciprocal genetic exchange, resulting in strict bivalent formation. *Ph1* determines the pattern of premeiotic alignment (see Vega and Feldman 1998, Wilson et al. 2005).

Barbosa and Vieira (1997) found variable configurations in the cells at diakinesis and metaphase I of the hybrids *P. edulis* f. *flavicarpa* + *P. amethystina*. Multivalent configurations did vary in frequency, and more than one quadrivalent was observed per cell. In the present study, the occurrence of only one quadrivalent per cell, with a relatively high but constant frequency, suggests that homeologous recombination was inhibited. However, a high meiotic stability is not of interest when the objective is to use them as a bridge for the transference of important genes from the wild. Some homeologous recombination as well as a certain level of pollen viability is essential (Binsfeld et al. 2001, Banga et al. 2003, Chen et al. 2004).

The presence of just a few univalents in the hybrid plants is expected considering the classical taxonomy (Killip 1938) and the modern classification proposed for the genus *Passiflora* (Ulmer and Macdougal 2004) that indicate the proximity of these species. Univalents and the various ways they behave throughout meiosis probably contributed to the formation of the micronuclei observed in the somatic hybrid microspores. The presence of univalents as micronuclei in tetrads was frequently associated with a reduction of vigour and fertility in hybrid plants (e.g. Cao et al. 2003) as reported previously for the somatic hybrids between *P. edulis* f. *flavicarpa* and *P. amethystina* (Barbosa and Vieira 1997).

The hypothesis to explain the occurrence of bridges is based on centromere misdivision of the univalents. Chromosomes segregate during division by interactions between their kinetochores and the plus-ends of spindle microtubules. Incorrect attachment of chromosomes to the spindle and transverse separation of the centromeres (Vega and Feldman 1998) lead to chromatin extending, which is frequently observed as a bridge without a fragment. In the past, Sears (1952) and more recently Vega and Feldman (1998) reported that the behaviour of these univalents is similar, in both meiosis I or II. In general, univalent behaviour is extremely variable, being observed in different configurations at different meiotic phases. Additionally, they can originate stable telocentrics and isochromosomes (Kaszás and Birchler 1996).

Finally, the more irregular the meiotic behaviour, the lower was the pollen viability. This correlation was also observed in previous studies (Barbosa and Vieira 1997). Although the hybrids often flower and produce viable pollen grains, they do not set fruit. Both parents are autoincompatible species and when they were artificially crossed with the parental species (theoretically, $2n = 4x = 36 \times 2n = 2x = 18 \rightarrow 2n = 3x = 27$), few seeds were obtained (data not shown). This information corroborates the cytogenetic findings, and suggests that several flowers should be pollinated in each cycle of a backcross-based breeding programme.

In practical terms, data here provided are useful for breeding programmes aiming at gene transfer, such as disease resistance genes. The levels of pollen viability and multivalent pairing in the hybrids suggest that these materials can be used as a bridge for gene transfer or even as rootstocks due to their compatibility to *P. edulis* f. *flavicarpa* (data not shown). One of the arguments used to inhibit the commercial production of transgenic material, namely species that are insect-pollinated, includes transgene escape to all related species. Much of this concern focuses on the expectation that if wild species or crops acquire transgenes promoting resistance to pests or diseases, they could invade or persist in agricultural areas or natural ecosystems (e.g. Armstrong et al. 2005). Transgeny should not be discarded in *Passiflora*, but the commercial varieties are indeed cultivated in proximity to their wild relatives. Therefore, one may suggest that most of the yellow passion-fruit-breeding efforts aiming at gene transfer should rely on sexual hybridization and even protoplast fusion procedures.

Acknowledgements

The authors are grateful to the Brazilian Institutions FAPESP and CNPq. M.M. receives a PRODOC/CAPES fellowship.

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